# Cell Surface P- and E-Selectin Support Shear-Dependent Rolling of Bovine γ/δ T Cells[1]

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The vascular selectins P- and E-selectin are inducible adhesion proteins expressed by endothelial cells that have been shown to support shear-dependent rolling of myeloid cells. This interaction is thought to be a prerequisite event for subsequent steps, such as tight adhesion/aggregation and transendothelial cell migration, involved in the accumulation of leukocytes into tissues. Certain lymphocyte subsets have also been shown to bind the vascular selectins, but the importance of this interaction in mediating shear-dependent rolling, as described for myeloid cells, has not been demonstrated. We expand on our earlier observation that bovine γ/δ T cells bind E-selectin by showing that this interaction leads to a reproducible rolling event in assays done under shear forces that approximate those that occur in vivo. E-selectin, expressed by L cell transfectants or cytokine-stimulated human and bovine endothelial cells, equally supports the shear-dependent rolling interaction. The lymphocyte adhesion proteins L-selectin, CD44, and CD2 do not contribute to this event. Neuraminidase treatment of the γ/δ T cells or addition of EDTA to the assay completely blocks the rolling interaction. We further show for the first time that P-selectin expressed by thrombin-activated platelets or a soluble P-selectin/human Ig chimera specifically binds γ/δ T cells. The P-selectin interaction is similar to the rolling event mediated by E-selectin—it requires divalent cations and sialic acid on the lymphocyte, it lacks involvement of L-selectin and CD44, and rolling occurs under physiologic shear conditions. These results provide the documentation that the vascular selectins can support shear-dependent rolling of a lymphocyte subset and that P-selectin mediates the adhesion of γ/δ T cells. *The Journal of Immunology*, 1994, 153: 3917.

Interactions between leukocytes, platelets, and endothelial cells at sites of vascular damage are required for effective host inflammatory responses. Selectins are a new, three-member family of adhesion proteins that are required for many of these interactions. P-selectin is an inducible adhesion protein expressed by thrombin-activated platelets or endothelial cells that has been shown to support adhesion of myeloid cells, such as neutrophils. E-selectin, expressed on cytokine-stimulated endothelial cells, is a second vascular selectin that binds circulating leukocytes. L-selectin, which interacts with ligands on endothelial cells, represents the only selectin expressed by leukocytes (1–4). Selectins are characterized by an N-terminal C-type lectin domain, followed by an epidermal growth factor-like region and multiple short consensus repeats. All three selectins also have conventional transmembrane domains and short cytoplasmic tail regions. The lectin activity of each selectin is important in function, and their carbohydrate binding specificities have been well characterized (1–4).

Selectins mediate neutrophil rolling interactions along inflamed vessels in both in vivo and in vitro assays done under flow (5–12). Purified P-selectin, coated onto glass or plastic surfaces, supports neutrophil rolling at wall shear stresses of 1.8 dynes/cm$^2$ (6). E-selectin cDNA-transfected L cells or purified E-selectin itself also support neutrophil rolling in vitro (11, 12). Abs to L-selectin and soluble selectin-Ig chimeras can block shear-dependent neutrophil rolling on inflamed venules (7–9). Transfection of L-selectin cDNAs into L-selectin-negative cell lines confers the capacity to roll in vivo, which is dependent on the cytoplasmic tail of L-selectin

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(10). It has been proposed that selectin-mediated rolling slows the leukocyte down from the flow of blood, allowing time for the cell to respond to activating agents released from the inflamed tissue, which in turn induces firm, static adhesion mediated by other adhesion molecules, such as the integrins (5, 13).

Originally, E- and P-selectin were characterized as myeloid cell adhesion molecules; however, recently, certain T cell subsets have been shown to interact with each of them (14–18), although the nature of the lymphocyte/P-selectin interaction is not as well defined as the E-selectin/lymphocyte interaction. T cells, which exhibit a memory phenotype and express the cutaneous lymphocyte-associated Ag recognized by the HECA 452 mAb, readily bind E-selectin (14, 15, 17). As shown for myeloid cells, E-selectin supports T cell binding via recognition of sialylated carbohydrates expressed by the leukocyte (19). The exact nature of the T cell carbohydrates and the structures which present the carbohydrates to E-selectin has not been fully defined. Whether the lymphocyte/vascular selectin interaction leads to a rolling event as described for myeloid cells has not been reported. Therefore, it is not known whether lymphocytes follow the multistep model of extravasation, outlined above for myeloid cells, to gain access into inflammatory lesions.

We have recently found that bovine γ/δ T cells represent another population of lymphocytes that avidly bind E-selectin (20). Their interaction with P-selectin has not been tested. Here, we used this model system to test whether vascular selectins could support shear-dependent rolling interactions of a lymphocyte. Specifically, we determined 1) whether E-selectin supports shear-dependent rolling of bovine γ/δ T cells, and 2) whether P-selectin supports a γ/δ T cell interaction like E-selectin. We found that both E-selectin cDNA transfectants and cytokine-stimulated endothelial cells supported shear-dependent rolling of γ/δ T cells under flow rates that approximate those that occur in vivo. The γ/δ T cells avidly bound thrombin-activated platelets via P-selectin, and a P-selectin/human Ig chimera bound specifically to γ/δ T cells. As seen with E-selectin, P-selectin supported shear-dependent rolling of the γ/δ T cells. Abs directed toward either E- or P-selectin blocked the rolling interactions in the in vitro assays, as did EDTA or neuraminidase treatment of the leukocyte. Abs directed toward adhesion molecules expressed by the T cells (L-selectin, CD44, and CD2) had no effect on rolling on either E- or P-selectin. These results provide the first demonstration that cell surface P- and E-selectin can support shear-dependent rolling of a specific lymphocyte subset.

## Materials and Methods

### Animals

BALB/c mice, ranging in age from 6 to 12 wk, were used as a source of cells and tissues. The mice were housed in the small-animal facility at Montana State University. One- to three-wk-old calves, which were housed in the Montana State University large-animal facility, were used as sources of peripheral blood and inflamed tissues.

### Cell preparations

Leukocytes were harvested from the peripheral blood of cattle as previously described (20). Briefly, blood was collected into heparin anticoagulant tubes, diluted 1:2 with warm HBSS, underlayed with Histopaque 1077, and centrifuged at 2300 rpm for 30 min at room temperature. Mononuclear cells were collected from the Histopaque/plasma interface. A lymphocyte preparation was prepared for the rolling assays by incubating the mononuclear cells on plastic for 1 h and collecting the nonadherent cells. In some experiments, γ/δ T cells were purified by a 30 min incubation on monolayers of E-selectin cDNA-transfected L cells cultured to subconfluency in T175 flasks. After the incubation period, the γ/δ T cells were removed from the monolayer by incubation in 1 mM EDTA. An additional adhesion step to plastic was done to reduce the number of monocytes in the preparation. This procedure yielded a population >90% γ/δ T cells.

Platelets were isolated from plasma samples, which had previously been platified of leukocytes, by centrifugation at 300 × *g*. The platelets were resuspended in HBSS and kept on ice until used in the capillary tube shear assay described below. Bovine and human umbilical cord endothelial cells (BUVECs[3] and HUVECs, specifically) were isolated by collagenase digestion of the lumenal surface of cords, and cultures were established as described previously (21). Only endothelial cells from the fourth passage or less, which were low density lipoproteins and factor VIII positive, were used in functional analyses. L cells stably expressing human E-selectin cDNA, previously described in Kishimoto et al. (22), were also used. Abbassi et al. (12) have recently shown that these cells support shear-dependent rolling of neutrophils. The original transfectant cultures were subcloned by limiting dilution and screened for a clone that was 100% E-selectin positive and exhibited a high, unimodal level of expression. The E-selectin expression on these cells was very stable, which led to greater consistency in the functional assays described below.

### Reagents and mAbs

Type I collagen (Collaborative Research, Inc.), bovine thrombin (Sigma Chemical Co., St. Louis, MO), and neuraminidase (Sigma Chemical Co.) were used. Human rTNF-α and rIL-1β, which cross-react in cattle, were gifts of E. Amento, Genentech (South San Francisco, CA). The following mAbs were used: DREG 56, which is a mouse IgG1 that recognizes and blocks the function of human and bovine L-selectin (23, 24); EL-246, which is a mouse IgG1 that recognizes both human and bovine E- and L-selectin (25); CC42, which is a mouse IgG1 that recognizes bovine CD2; ILA29, which is a mouse IgG1 that recognizes the bovine WC1 (T19) Ag on γ/δ T cells (26); HERMES 3, which is a mouse IgG1 that recognizes human and bovine CD44 (24); EL-81, which is a mouse IgG1 that recognizes a nonfunctional epitope on E-selectin (20, 25); 12.2, which is a mouse IgG1 that recognizes and blocks the function of P-selectin (A. Warnock and E. C. Butcher, unpublished observations); and GSEC 435, which is a mouse IgG1 that recognizes an activation Ag expressed by thrombin-treated platelets (M. A. Jutila, unpublished observations). We have confirmed the reactivity of the 12.2 Ab. It specifically recognizes a 140 kDa surface protein in platelets and P-selectin cDNA transfectants, and the Ab blocks neutrophil aggregation with thrombin-activated platelets (M. A. Jutila, unpublished observations). We also confirmed the reactivity of the CC42 and ILA29 mAbs, which recognize surface markers distinct from the TCR on bovine lymphocytes that distinguish the majority of αβ vs γ/δ T cells, respectively (26–28). Specifically, CC42 did not stain B cells (surface $Ig^+$, $CC21^+$), and only a very few γ/δ T cells; whereas, ILA29 stained $CD4^-$, $CD8^-$, $CD2^-$, γ/δ TCR-positive cells. All Abs were partially purified by ammonium sulfate precipitation of serum-free supernatant fluids, and at 10 μg/ml concentrations contained <2 pg LPS, as detected by gel agglutination assay (Sigma). A human P-selectin/human Ig chimera, produced as previously described (29, 30), was also used. The chimera was derived from human P-selectin and the CH2 and CH3 domains of human γ-globulin. The chimera is produced much like a mAb and provides a soluble source of

[3] Abbreviations used in this paper: BUVEC, bovine umbilical cord endothelial cell; FBS, fetal bovine serum.

functional P-selectin. Previous studies show that the chimera specifically binds selectin carbohydrate ligands, binds to a ligand on neutrophils, and inhibits the accumulation of rat neutrophils in sites of inflammation in vivo (29–31). A CD4/Ig chimera (32) was used as a negative control.

### *Immunofluorescence staining and flow cytometric analysis*

Immunofluorescence staining of leukocytes was conducted as described (33, 34). Briefly, $1 \times 10^6$ cells were initially incubated in 2% rabbit serum for 10 min on ice to block Fc receptors. The cells were washed and then incubated with primary Ab at 50 $\mu$g/ml (or undiluted culture supernatant) for 20 min on ice. After washing, bound Abs were revealed by incubation with phycoerythrin-conjugated $F(ab')_2$ goat anti-mouse Ig (Jackson) at a 1:250 dilution in 5% fetal bovine serum (FBS) in DMEM. Cells were also stained with the P-selectin/Ig chimera and revealed by a FITC-conjugated anti-human Ig second stage (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Two-color staining was done by combining the analyses of the mouse mAbs and the P-selectin chimera. Flow cytometric analysis was made on a FACScan (Becton Dickinson, Mountain View, CA) as described (33, 34). Isotype-matched negative controls were used to evaluate the level of background staining, which was subtracted from most analyses or included as overlays of the positive histograms. Data were collected from 10,000 to 50,000 cells.

### *Capillary tube shear-dependent rolling assay*

We have previously described a new approach for measuring the interaction of leukocytes with cells expressing adhesion proteins under controlled shear forces that approximate those that occur in vivo (35).[4] This assay employs capillary tubes instead of planar chambers as the matrix for the adhesive substrate, and has been very useful in the analysis of shear-dependent neutrophil/endothelial cell interactions (35). The tubes are integrated into a free-flowing circulatory loop into which cells and inhibitors can be sequentially infused over time. This system can be set up to examine leukocyte interactions with adherent cells under flow conditions similar to those described in vivo. An advantage of this model is that inhibitors can be directly infused into the loop, in addition to pretreatment of the cells before the assay. Rolling interactions can be established first and inhibitors infused later, which avoids potential artifacts associated with pretreatment incubation times and washing steps (35).

For the studies described here, control L cells or L cells expressing recombinant human E-selectin, or HUVEC, were grown to confluency on the internal surface of sterile glass 1.4-mm capillary tubes (Drummond Scientific, Broomall, PA) previously coated with collagen type I or medium alone. Four hours before the rolling assays, endothelial cells were treated with 10 ng/ml IL-1$\beta$ or 1 $\mu$/ml TNF-$\alpha$, which we have shown to induce maximal E-selectin expression. Stimulation was not required for the L cell transfectants. Tubing (internal diameter, 1.5 mm) was attached to each end of the capillary tube to form a closed system in which fluid and cells could be recirculated by using a variable speed peristaltic pump containing six rollers to reduce pulsation; length and volume of the system was approximately 105 cm and 3 ml, respectively. The capillary tube was mounted on a 37°C-heated stage of an inverted microscope modified for video microscopy. Purified bovine mononuclear cells were injected into the system at a $2 \times 10^6$ cell/ml concentration in DMEM plus 2% FBS and 20 mM HEPES. A reproducible rolling interaction (defined as cells that turn a complete rotation while in contact with the monolayer) on the cytokine-activated endothelial cells and the E-selectin cDNA-transfected L cells, which did not occur on unactivated endothelial cells or nontransfected L cells, was detected under flow rates of 10 to 50 mm/s (value obtained by measuring the length of time for a complete rotation through the loop system). Noninteracting cells remained within the flow of the fluid and were not readily seen in the videotapes because of the high velocities. The Hagen-Poiseuille equation predicts that these flow rates represent shear forces of 0.6 to 3.0 dynes/cm$^2$ in this system. The number of mononuclear cells rolling on the activated endothelial cells or E-selectin cDNA-transfected L cells at 30-s intervals was determined by analyzing individual frames of the videotape recording. Aggregation of cells was usually not a problem with the $\gamma/\delta$ T cells, but when aggregates were detected, they were excluded from the analyses. Only the interactions of single, isolated lymphocytes were recorded. Data were recorded as the number of rolling cells within the field of view vs time.

To test the effects of various inhibitors, rolling events were established; then, mAbs at 50 $\mu$g/ml concentrations, neuraminidase (1 U/ml in pH 6.5 acetate buffer), pH 6.5 acetate buffer alone (negative control), or 5 mM EDTA were infused into the loop system. In some instances, the lymphocytes were treated with the inhibitors before injection into the shear assay. Shear forces of 2 to 3 dynes were used in all blocking assays. The effect of treatments on the number of rolling cells was then determined by analyzing the videotapes.

The phenotype of the rolling cells was determined by first establishing a rolling interaction, rapidly perfusing the system with 1.5% glutaraldehyde, which fixed the rolling cells to the endothelial cell or L cell monolayers, and then staining with lymphocyte lineage-specific mAbs by immunoperoxidase. The fixation procedure caused retraction of many of the adherent cells bound to the capillary tubes. The immunoperoxidase stains were done using the procedures of a standard kit employing biotin anti-mouse second stage and avidin peroxidase (Histoprobe; Tago Immunologicals, Burlingame, CA).

We also established conditions for measuring the interaction of leukocytes with thrombin-activated platelets in the capillary tube shear assay. Platelets isolated from either human or bovine plasma were coated at $1.5 \times 10^9$ cells/ml onto the internal surface of the collagen-coated capillary tubes and allowed to attach for 15 to 30 min. In some instances, the bound platelets formed complete monolayers on the capillary tubes; however, in most experiments, the monolayers were irregular, exhibiting small gaps that exposed the surface of the capillary tube. Thrombin (1 U/ml for 10 min) was used to activate the bound platelets. After incubation and the establishment of the bound platelets, tubing was attached to each end of the capillary tube and the assay was set up as described above. Bovine mononuclear cells were injected at $2 \times 10^6$ cells/ml in DMEM plus 2% FBS and 20 mM HEPES. Increasing levels of shear were applied to the system, and the interaction of the leukocytes with the bound platelets was monitored by videomicroscopy, as described above. Under shear forces similar to those used in the endothelial cell or L cell assays, we found that bovine mononuclear cells avidly rolled on the bound platelets. Rolling was only observed over the surface of the platelets and did not occur in the gaps of the monolayers: establishment of a complete or irregular monolayer of platelets did not alter the rolling behavior of the leukocytes, except to affect the rolling speeds. The specificity of the rolling interaction was then determined by infusion of mAbs and other agents as described above. The phenotype of the rolling cell was also determined by immunoperoxidase staining after glutaraldehyde fixation. As was seen with the bound L cell transfectants, the fixation procedures caused retraction of many of the platelet aggregates, which exposed multiple gaps in the monolayer.

## Results

### *E-selectin cDNA-transfected L cells support rolling of $\gamma/\delta$ T cells*

In earlier studies we found that bovine $\gamma/\delta$ T cells avidly bound E-selectin cDNA-transfected mouse L cells (20). A new subclone of the transfectants, which expressed uniform levels of E-selectin similar to those on cytokine-activated endothelial cells (data not shown), was grown on the internal surface of glass capillary tubes and the shear-dependent rolling assay was set up as described in *Materials and Methods*. We found that the E-selectin transfectants readily supported rolling of bovine lymphocytes under shear conditions similar to those defined in vivo and in other in vitro shear models (6) (0.6 to 3.0 dynes/cm$^2$). Rolling was continuous across the monolayer and exhibited an average speed of 6.8 $\pm$ 2.5 $\mu$m/s (analysis of the speed of 10 separate cells in a typical experiment done at

[4] R. F. Bargatze, S. Kurk, E. C. Butcher, and M. A. Jutila. 1994. Neutrophils roll on adhered neutrophils via L-selectin on the rolling cell. *J. Exp. Med. In press.*

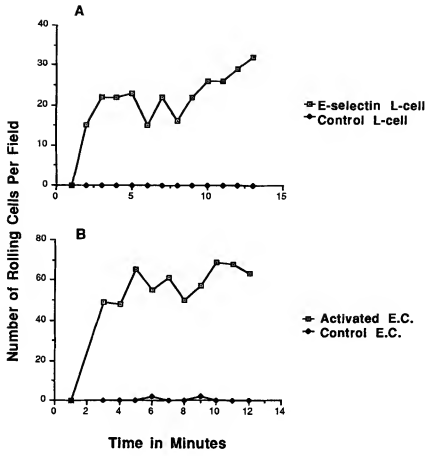


**FIGURE 1.** Bovine lymphocytes roll on monolayers of E-selectin cDNA-tranfected L cells and IL-1-stimulated endothelial cells. In *A*, the rolling of bovine lymphocytes on E-selectin cDNA-transfected and control L cells is compared. In *B*, the rolling of bovine lymphocytes on 4-h IL-1 (10 U/ml)-stimulated and control BUVEC (E.C.) is compared. The data are from representative experiments which were repeated more than three times. The circulatory loop assays were set up as described in *Materials and Methods*. The lymphocyte suspensions were injected into the loop system at the 1-min time point. The number of rolling cells per field of view was then determined at 1-min intervals.

approximately 2 dynes/cm$^2$), which reflected >1000-fold reduction in velocity from the flow of the system. Figure 1*A* shows a plot of the number of cells actively rolling on the E-selectin transfectant monolayer vs time at a shear force of approximately 2 dynes/cm$^2$. In this one experiment, maximal rolling occurred within minutes after injection of the lymphocytes into the circulatory loop, and was maintained for more than 13 min. In other experiments, rolling interactions were maintained and analyzed for more than 45 min. Rolling decreased when shear was increased above 3 dynes/cm$^2$ (data not shown). As a control, we found that lymphocytes did not roll on the non-transfected L cells (Fig. 1*A*).

A rolling interaction also occurred between bovine lymphocytes and 4-h IL-1-stimulated BUVEC cultured on the internal surface of the capillary tubes (Fig. 1*B*). Endothelial cells treated in such a manner expressed high levels of E-selectin (data not shown). Bovine lymphocytes did not roll on unstimulated endothelial cells (Fig. 1*B*). Similar results were obtained with bovine lymphocytes on cytokine-stimulated HUVEC (data not shown).

To determine the phenotype of the cells that rolled on the E-selectin monolayer, the rolling cells were fixed to the transfectants by rapidly perfusing the system with 1.5% glutaraldehyde. The tubes were then stained with Abs directed against the majority of $\alpha/\beta$ (CD2) and $\gamma/\delta$ T cells. More than 90% of the bound lymphocytes were $\gamma/\delta$ T cells (ILA29+), whereas the cells make up only 41 ± 12.2% (analysis of the cells from 12 animals) of the isolated lymphocytes. Less than 1% of the cells were CD2 positive. Figure 2*A* shows a representative immunoperoxidase stain of the $\gamma/\delta$ T lymphocytes attached to the monolayer. The remaining cells were predominantly monocytes (Fig. 2*B*).

A function-blocking, anti-E- and anti-L-selectin mAb (EL-246, 50 μg/ml) blocked the rolling interaction within 60 to 90 s after being injected into the circulatory loop, whereas a nonblocking anti-E-selectin Ab (EL-81, 50 μg/ml) had little effect (Fig. 3*A*). Other anti-E-selectin blocking mAbs (EL-26, EL-112) also blocked, whereas anti-P-selectin mAb (12.2) did not (data not shown). Figure 4 shows video stills of the effects of the EL-246 mAb that dramatically illustrate the blocking of the rolling event. These results show that the rolling interaction is mediated by E-selectin but not P-selectin.

Antibodies to a number of different leukocyte adhesion molecules were also tested to determine whether they contributed to the rolling of $\gamma/\delta$ T cells on the E-selectin transfectants. The $\gamma/\delta$ T cells were treated with saturating levels

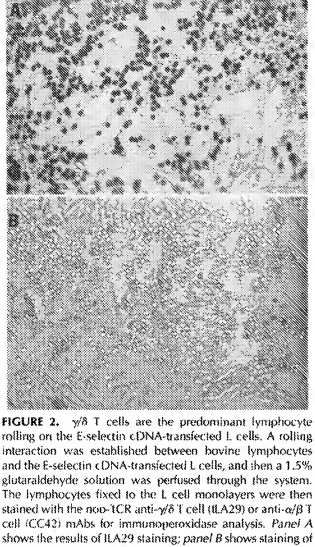


**FIGURE 2.** γ/δ T cells are the predominant lymphocyte rolling on the E-selectin cDNA-transfected L cells. A rolling interaction was established between bovine lymphocytes and the E-selectin cDNA-transfected L cells, and then a 1.5% glutaraldehyde solution was perfused through the system. The lymphocytes fixed to the L cell monolayers were then stained with the non-TCR anti-γ/δ T cell (tLA29) or anti-α/β T cell (CC42) mAbs for immunoperoxidase analysis. *Panel A* shows the results of tLA29 staining; *panel B* shows staining of CC42. The fixation and staining procedure caused slight retraction and loss of some of the bound L cells. Note that most of the lymphocytes fixed to the monolayer stained with tLA29, whereas in this example few if any cells stained with CC42 (other experiments showed occasional CC42 positive cells). The curvature of the tubes caused the blurring effects of cells in the upper and lower portion of the figure. Staining with isotype-matched negative control Abs was completely negative (data not shown).

of mAbs directed against CD44 (HERMES 3 mAb), CD2, and L-selectin (DREG 56); however, none of these Abs affected rolling (Fig. 3*A*). In contrast, neuraminidase treatment (1 U/ml) of the γ/δ T cells, which removes terminal sialic moieties, did block their ability to roll on E-selectin (Fig. 3*B*). As described in our earlier report (20), EDTA treatment also blocked the interaction (data not shown).

### *Thrombin-activated platelets support the rolling of γ/δ T cells*

We next determined whether thrombin-activated platelets, which should express P-selectin, could support the rolling of γ/δ T cells. The thrombin treatment induced significant levels of P-selectin on the surface of the platelets, as determined by staining with an anti-P-selectin (12.2) mAb (Fig. 5*A*). GSEC 435, which recognizes a platelet Ag distinct from P-selectin (M. A. Jutila, unpublished observations), also specifically stained the thrombin-activated platelets (Fig. 5*B*). The thrombin-activated platelets were coated onto the internal surface of collagen-coated capillary tubes. The platelets formed incomplete monolayers of aggregates and single cells on the internal surface of the capillary tubes.

We next found that bovine PBMCs avidly roll on monolayers of thrombin-activated bovine or human platelets. Rolling did not occur on the collagen-coated glass surface exposed in gaps in the monolayers. The rolling speeds of the cells on a typical platelet monolayer were 16.8 ± 7.0 μm/s (analysis of 10 separate cells at 2 dynes). The speeds varied greatly between experiments because of differences in the continuity of the monolayers, but in all cases, they demonstrated a considerable reduction in velocity from the flow of the system. Immunoperoxidase analysis of the rolling cells, done as described above, demonstrated that 80 ± 13% of the rolling cells on bovine platelets were γ/δ T cells, whereas 11 ± 6% were CD2 positive. Figure 6 shows an example of an immunoperoxidase stain for γ/δ T cells on the platelet monolayer. A similar percentage of bovine γ/δ T cells rolled on thrombin-activated human platelets (data not shown). Anti-human P-selectin mAb was then used to determine whether human P-selectin supported this interaction. As shown in Figure 7*A*, the 12.2 anti-P-selectin mAb effectively inhibited the rolling interaction in two different experiments. In contrast, the isotype-matched control Ab GSEC 435, which also recognized the activated platelets (Fig. 7*A*), had no effect. Because the 12.2 mAb only recognizes the platelets and not the leukocytes in this system and no endothelial cells are present, these results clearly show that platelets can support shear-dependent rolling of a lymphocyte subset.

We then tested the effect of a number of different mAbs against surface Ags on the γ/δ T cells. DREG 56, EL-246, and HERMES 3 had no effect on the rolling of γ/δ T cells on activated platelets (Fig. 7*B*). EL-246 also blocks E-selectin, and its lack of effect is additional support for the specificity of this interaction. As was shown for rolling on E-selectin above, neuraminidase and EDTA treatment of the lymphocyte completely blocked the interaction (data not shown).

As a final means of confirming that P-selectin supports the shear-dependent rolling of γ/δ T cells, we used a P-selectin/Ig chimera, which has been described in detail in other reports (29, 30, 31). Briefly, the chimera provides a

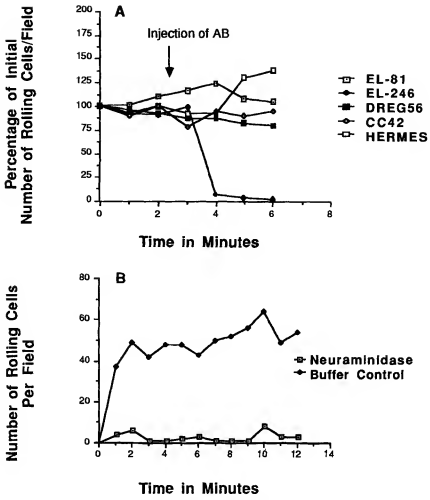


**FIGURE 3.** Effect of Abs directed against different adhesion molecules and neuraminidase on the rolling of γ/δ T cells on E-selectin cDNA-transfected L cells. In these experiments, purification procedures were done, as described in *Materials and Methods*, to prepare leukocyte suspensions which were >90% γ/δ T cells. In *A*, a rolling interaction was established between the γ/δ T cells and the E-selectin transfectants, and then various mAbs were injected into the loop system. The effect of the mAbs was then followed over time. Results are presented as a percentage of the initial number of rolling cells. In *B*, γ/δ T cells were treated with 1 U/ml neuraminidase or its acetate buffer control for 15 min and then injected into the loop system. The number of rolling cells per field of view was then followed over time. Results are from representative experiments that were repeated more than three times and are essentially the same as our previous studies (20).

soluble source of P-selectin that can be used in flow cytometric and functional analyses. We first compared the staining of the chimera on γ/δ vs α/β T cells by flow cytometry. Figure 8 shows histograms of the staining of γ/δ and α/β T cells revealed by the ILA29 and CC42 mAbs. The chimera specifically stained >70% of the γ/δ T cells (Fig. 8*A*), whereas no reactivity was seen with α/β T cells (Fig. 8*B*). A control chimera construct (human CD4/Ig) did not bind the γ/δ T cells (data not shown). The binding of the chimera to the γ/δ T cells was inhibited by EDTA (Fig. 8*A*).

We next tested whether γ/δ T cells, pretreated with the P-selectin chimera, could roll on the thrombin-activated human or bovine platelets. As shown in Figure 9, the P-selectin chimera-treated γ/δ T cells showed a diminished capacity to initiate rolling; however, over time, rolling did begin to occur. The CD4/Ig chimera had little effect on the initiation of rolling (Fig. 9). These results suggest that the P-selectin chimera does block rolling interactions with P-selectin on activated platelets, but that it may exhibit a lower affinity for the leukocyte than does native P-selectin and may be competed from the leukocyte surface over time.

## Discussion

In recent years, it has become clear that the migration of leukocytes from the blood into tissues is quite complex. Studies with myeloid cells suggest that at least three steps are required: 1) a reversible interaction, represented by rolling of the leukocyte along the vascular wall; 2) stopping and tight adhesion; and 3) migration of the leukocyte across the endothelial barrier (11, 13). It is likely that a similar sequence of events is required for the migration of lymphocytes into various tissues. Indeed, Bargatze and Butcher (36) have recently shown that lymphocyte migration into mouse Peyer's patches involves a reversible rolling interaction, a pertussis toxin-inhibitable signaling event, and activation-dependent tight adhesion. However, the role of the three-step model in lymphocyte extravasation in defined in vitro systems has not been previously reported.

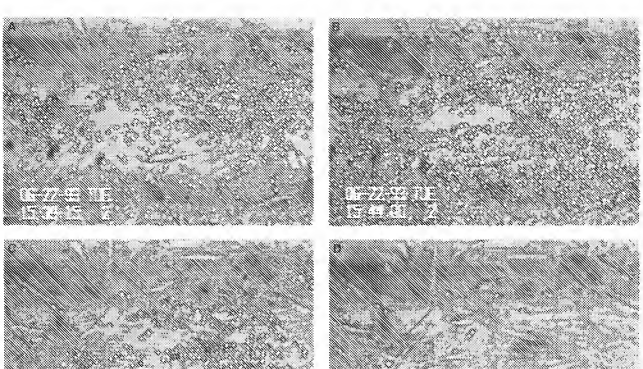

**FIGURE 4.** Video stills demonstrating the effect of the EL-246 anti-selectin mAb on the rolling of γ/δ T cells on E-selectin cDNA transfectants. An Ab blocking experiment was done, as described in Figure 3, using the EL-246 anti-selectin mAb. Stills of the videotape analysis before and immediately after the injection of the mAb are shown. *Panel A* shows the rolling of purified γ/δ T cells on a monolayer of E-selectin cDNA-transfected L cells. The EL-246 mAb was injected at the time point indicated in *panel B*. *Panels C* and *D* show the effects of the mAb 50 and 60 s, respectively, after its injection into the loop assay. All of the cells removed from the field of view after the injection of EL-246 were originally rolling from right to left. EL-246 had no effect on the few tightly adhered lymphocytes (see the few remaining lymphocytes in *panel D*).

E-selectin supports the adhesion of certain subsets of lymphocytes, and this interaction has been proposed to be important in regulating the migration of these cells into some sites of inflammation (14, 15, 17). E-selectin mediates reversible, shear-dependent rolling interactions of myeloid cells—the first step of extravasation. We show that recombinant human E-selectin expressed in mouse L cells or the native molecule expressed by cytokine-activated endothelial cells effectively supports shear-dependent rolling of bovine γ/δ T cells. This interaction requires divalent cations and sialic acid moieties expressed on the surface of the lymphocyte. Interestingly, Abs directed against CD44, CD2, L-selectin, and also CD 18 (analyzed in another study) had no effect on rolling. In other studies, we have found that human lymphocytes (HECA 452$^+$ cells) also roll on E-selectin L cell transfectants in the capillary tube shear assay (R. F. Bargatze and M. A. Jutila, unpublished observations). Others have demonstrated this as well (L. J. Picker, personal communication). Thus, as described for myeloid cells, E-selectin supports a reversible shear-dependent rolling interaction of lymphocytes, which does not involve CD44 or CD18. Interestingly, L-selectin also has no apparent role in the lymphocyte interaction, which is in direct contrast to recent observations of neutrophil rolling on E-selectin (12, 37). This is significant because L-selectin is apparently expressed on the microvilli of lymphocytes (9), as seen with neutrophils (37); thus, it is in appropriate physical proximity to bind E-selectin. What accounts for the difference in the use of L-selectin on neutrophils vs lymphocytes is unknown, but it may be related to the carbohydrate decoration of the molecules, as proposed by Picker et al. (37).

P-selectin represents another vascular selectin that supports leukocyte rolling (6). In addition to expression on thrombin-activated endothelial cells, P-selectin is also expressed by thrombin-activated platelets. Here, we tested for the first time whether γ/δ T cells bind P-selectin, and, if they do, whether P-selectin supports a rolling interaction

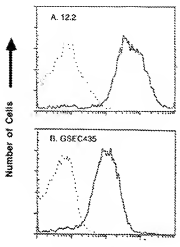


**FIGURE 5.** The 12.2 and GSEC 435 mAbs specifically stain thrombin-activated platelets. Human platelets were prepared and activated for 10 min with 1 U/ml thrombin as described in *Materials and Methods* and then stained for flow cytometric analysis with the 12.2 and GSEC 435 mAbs. *Panel A* shows the staining of 12.2 on unactivated (dotted line) and activated (solid line) platelets. *Panel B* shows the staining of GSEC 435 on the same cells. Background staining with isotype-matched negative control mAbs exhibited mode fluorescence values of <10.

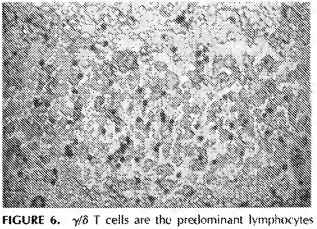

**FIGURE 6.** γ/δ T cells are the predominant lymphocytes rolling on thrombin-activated platelets. A rolling interaction was established between bovine lymphocytes and thrombin-activated platelets bound to the lumenal surface of the capillary tube, and then a 1.5% glutaraldehyde solution was perfused through the system. The lymphocytes fixed to the platelet monolayers were then stained with an anti-γ/δ T cell (BLA29) mAb for immunoperoxidase analysis. The fixation and staining procedure caused retraction and loss of some of the platelet aggregates, revealing gaps in the monolayer. Note that most of the lymphocytes fixed to the platelet aggregates stained with the anti-γ/δ T cell mAb. The curvature of the tube caused the blurring effects of cells on the upper and lower portions of the figure. Staining with isotype-matched negative control Abs was completely negative (data not shown).

as seen with E-selectin. We used two approaches to do these analyses. In the first, we established monolayers of activated platelets on the internal surface of capillary tubes. In many instances, the platelets formed almost complete and intact monolayers (see Fig. 6, for an example). We showed that immobilized, thrombin-activated platelets from humans or cattle effectively supported the rolling of γ/δ T cells. The interaction was very similar to E-selectin-mediated rolling. Monoclonal Abs directed against P-selectin blocked the rolling interaction by >90%, whereas another Ab directed against an undefined activation-induced platelet surface Ag had no effect. As described for the E-selectin interaction, P-selectin-dependent rolling required divalent cations and neuraminidase treatment of the lymphocyte blocked the interaction. Antibodies directed against CD44, E-selectin, and L-selectin were without effect. An interesting difference between P- and E-selectin was that α/β T cells rolled in greater numbers on the former.

Our second means of analyzing the binding of γ/δ T cells to P-selectin used a P-selectin/Ig chimera that has been used in other studies of leukocyte/P-selectin interactions. The specific P-selectin chimera used in this study has been shown in other systems to bind human myeloid cells (neutrophils and monocytes) and block the accumulation of rat neutrophils at sites of inflammation (29, 30). We extended these studies in cattle by first showing that the chimera avidly binds bovine myeloid cells (data not shown). We then found that the chimera specifically stains γ/δ T cells by two-color flow cytometric analysis, an interaction sensitive to EDTA. Approximately 60 to 70% of circulating γ/δ T cells in newborn calves bound the P-selectin/Ig chimera, which is less than our analyses of the percentage of γ/δ T cells that can bind E-selectin. By sequentially passing a suspension of γ/δ T cells over E-selectin transfectants, we specifically removed >90% of the cells (20). This suggests that P- and E-selectin do not interact equally with γ/δ T cells.

Once we showed that the P-selectin/Ig chimera could bind γ/δ T cells, we determined that it could partially block the ability of the lymphocytes to initiate rolling on thrombin-activated human or bovine platelets. Interestingly, the γ/δ T cells treated with the chimera eventually did roll on the platelet monolayers. Because saturating levels of the chimera were not maintained in the loop system (the lymphocytes were pretreated), our results suggest that native P-selectin on the surface of the bound platelets exhibits a greater affinity for supporting cell adhesion than the soluble chimera.

In given experiments, γ/δ T cell rolling speeds on E- and P-selectin under physiologic shear force (2 dynes/

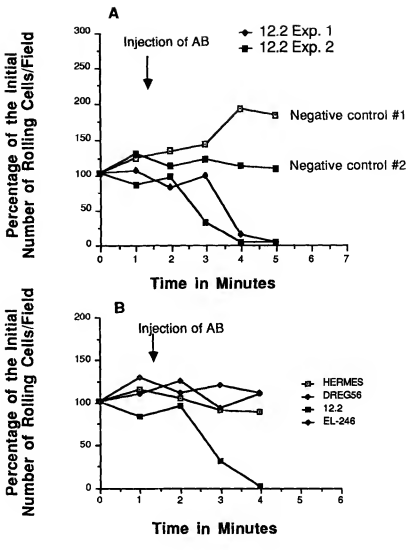


**FIGURE 7.** Anti-P-selectin mAbs, but not Abs directed against other adhesion molecules, block the rolling of γ/δ T cells on thrombin-activated platelets. A rolling interaction between purified γ/δ T cells on monolayers of thrombin-activated human platelets was established and then various mAbs were injected into the loop system. In *panel A*, the results of two different experiments demonstrating that the 12.2 anti-P-selectin mAb blocks the rolling interaction, are shown. In contrast, the GSEC 435 control mAb had little effect. *Panel B* shows that mAbs directed against CD44 (HERMES), L-selectin (DREG 56), or L- and E-selectin (EL-246) had no effect on the rolling of γ/δ T cells on the activated platelets.

$cm^2$) were approximately 6.8 and 16.8 μm/s, respectively. These speeds are affected by the density of the ligands on the tube and, thus, are difficult to relate in vivo and to other in vitro systems, but they are close to the range of rolling velocities identified for leukocytes along vessel walls in the animal (range 10 to 840 μm/s) (38). Importantly, these speeds represent a considerable reduction from the flow in our closed-loop system, which has center-line velocities between 10 and 50 mm/s. Thus, 1000-fold reductions in speed are attained via these interactions. Whether this dramatic slowing effect on the lymphocyte allows the appropriate signaling events leading to tight adhesion to take place, as predicted by the multistep model of extravasation, is yet to be determined.

The value of analyzing bovine γ/δ T cells vs human cells is that in vivo experiments can be done. We have found that bovine γ/δ T cells readily accumulate along the lumenal surface of inflamed vessels that express E-selectin in vivo (20). Recently, we have found that this interaction can be blocked in the animal by injection of anti-E-selectin mAbs (M. A. Jutila, unpublished observations); thus, our in vitro observations have correlated precisely with function in vivo. Furthermore, these studies provide the first direct in vivo evidence for the importance of a vascular selectin in controlling the homing of a lymphocyte subset. We are attempting a similar approach with P-selectin. In preliminary experiments, we injected 10 U thrombin into the skin of newborn calves. The treated tissue was harvested within 10 min after the thrombin injection—long before the expression of E-selectin occurs. Using immunoperoxidase analysis, we have found γ/δ T cells accumulated along the vessel walls at the thrombin injection site, although considerable variability has been seen between animals and between injection sites. As was done with E-selectin, we intend to determine whether the homing profile of γ/δ T cells can be altered by blocking P-selectin.

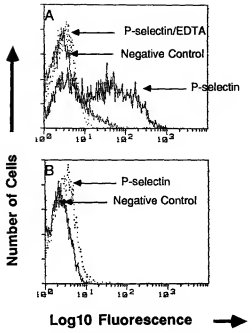


**FIGURE 8.** A P-selectin/human Ig chimera specifically stains bovine γ/δ T cells. Two-color immunofluorescence staining was done on bovine mononuclear cells using the P-selectin chimera and either an anti-γ/δ T cell mAb (ILA29) or an anti-α/β T cell mAb (CC42). A live gate was set to analyze only γ/δ T cells or α/β T cells. *Panels A* and *B* show the staining of the P-selectin chimera (P-selectin) on gated γ/δ T cells and α/β T cells, respectively. Negative control is the staining with the anti-human Ig second stage control. A similar level of background staining was seen with the CD4/Ig chimera control (data not shown). *Panel A* also shows that EDTA treatment blocks the staining of the γ/δ T cells by the P-selectin chimera (P-selectin/EDTA).

In addition to our in vivo studies, we are also pursuing the characterization of the nature of the selectin ligands expressed by γ/δ T cells. We have found that sialic acid-modified carbohydrates on the surface of γ/δ T cells are important in binding to both P- and E-selectin; as shown for all other leukocyte/selectin interactions (1, 3, 29, 39, 40). Interestingly, the bovine γ/δ T cell carbohydrates are antigenically distinct from the selectin carbohydrate ligands defined in humans. Antibodies directed against sialyl-Lewis$^x$ and the cutaneous lymphocyte Ag, which represent the human neutrophil and lymphocyte carbohydrates, respectively (19), cross-react in cattle; yet, they do not stain bovine γ/δ T cells (20). We have also demonstrated the importance of protein on the lymphocyte in presenting the carbohydrate ligands to E-selectin, much like what has been found for myeloid cells. However, as mentioned in the preceding discussion, unlike myeloid cells (22, 37), γ/δ T cells do not use L-selectin in their rolling interaction on E- or P-selectin (20).

To examine more thoroughly the nature of surface glycoproteins expressed by γ/δ T cells that bind E-selectin, we used E-selectin as an affinity reagent and specifically isolated a single 250-kDa glycoprotein from γ/δ T cell detergent lysates (20). The interaction of the 250-kDa glycoprotein with E-selectin is sensitive to EDTA and neuraminidase (20). This undefined molecule may be similar to previously described large m.w. glycoproteins expressed by other leukocytes and endothelial cells, which have been shown to be selectin ligands (41, 42). In particular, using an E-selectin chimera, Levinovitz et al. (42) isolated a single glycoprotein of 150 kDa from a mouse neutrophil cell line. In analyses of a mixed-mouse bone marrow population, the 150 kDa glycoprotein, as well as a 250-kDa molecule, were identified. The 250-kDa glycoprotein isolated by Levinovitz may be the same as the molecule we have isolated from bovine γ/δ T cells. We have not determined whether P-selectin also interacts with the 250-kDa molecule. Although the use of adhesion molecules as affinity reagents to analyze detergent lysates of target cells has been a powerful approach in identifying these new molecules, more analyses are needed to confirm that they actually mediate the cell–cell binding event and not simply interactions in solution. This is an important goal, because their characterization may lead to the development of novel inhibitors of lymphocyte migration.

Finally, our studies of the γ/δ T lymphocytes give insight into the molecular basis for the unique homing behavior of these cells. Unlike other naive lymphocytes, which selectively recirculate through secondary lymphoid organs, naive γ/δ T cells, much like memory lymphocytes, continuously recirculate through extralymphoid, epithelial-associated tissues and sites of inflammation (43). The recirculation of γ/δ T cells in these tissues suggests that they may serve as an important line of host defense against infectious agents. Indeed, recent Ab clearance studies have shown the γ/δ T cells to be important in the initial host response to *Listeria monocytogenes* and *Mycobacterium tuberculosis* infections, among others (44). It could be expected that the inflammatory reactions associated with these bacterial infections would lead to expression of either P- or E-selectin or both. Once this occurs, rapid mobilization of γ/δ T cells may ensue.

In summary, the use of a new, shear-dependent assay has shown that the two vascular selectins E- and P-selectin effectively support rolling of γ/δ T cells. These are one of the first studies to 1) clearly document a selectin-dependent lymphocyte rolling interaction, 2) show that P-selectin expressed by thrombin-activated platelets supports lymphocyte rolling, 3) demonstrate that bovine γ/δ T cells avidly bind P-selectin, and 4) analyze lymphocyte interactions in an animal model that allows for determination of the in vivo relevance of these events. These results define and provide a role for the first step of the multistep model of extravasation defined for myeloid cells in the migration of certain lymphocyte subsets into sites of inflammation.

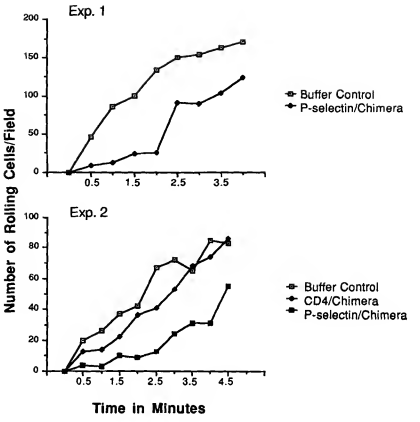


**FIGURE 9.** Pretreatment of γ/δ T cells with the P-selectin chimera reduces the onset of rolling on thrombin-activated platelets. The results of two experiments testing the effect of pretreating γ/δ T cells on their ability to roll on thrombin-activated platelets are shown. Purified γ/δ T cells were pretreated with medium alone or with 50 μg/ml concentrations of the P-selectin or CD4 chimeras for 20 min on ice and then injected into the loop system. In both experiments, γ/δ T cells pretreated with the P-selectin chimera exhibited lower numbers of rolling cells compared with the controls, particularly at the early time points (<5 min).

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